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**{Exhibit 37}**

Mosback, K., et al., "immobilized Coenzymes," Methods in Enzymology, Vol. XLIV: 859-887 (1976)

# [61] Immobilized Coenzymes

*By* KLAUS MOSBACH, PER-OLOF LARSSON, and CHRISTOPHER LOWE

The immobilization of coenzymes has received increasing attention over the last few years because they have several important applications: (1) as "active immobilized coenzymes," (2) as immobilized general ligands in affinity chromatography (this applies in particular to the various nucleotides such as AMP), and (3) a few reports have recently appeared in which they have been applied to more fundamental enzymological studies. The aspects of affinity chromatography have been covered in a recent volume of this series[1] and are not dealt with here. In this volume the main emphasis is on their use as active coenzymes together with a brief account of their application in basic enzymology. The methodological part will be centered around the various adenine nucleotides, $NAD^+$, $NADP^+$, ATP, ADP, whereas work on other coenzymes will be treated only in a summary fashion. The various aspects will be treated as outlined below: (1) synthesis of a number of adenine nucleotide coenzymes, (2) coupling to matrices, (3) coenzymic activity, (4) application in enzyme technology and analysis, (5) application in enzymological and protein studies, (6) other immobilized coenzymes, (7) general discussion.

## Synthesis of $N^6$-carboxymethyl- and $N^6$-[(6-aminohexyl) carbamoylmethyl] Derivatives of $NAD^+$, $NADP^+$, ATP, and ADP

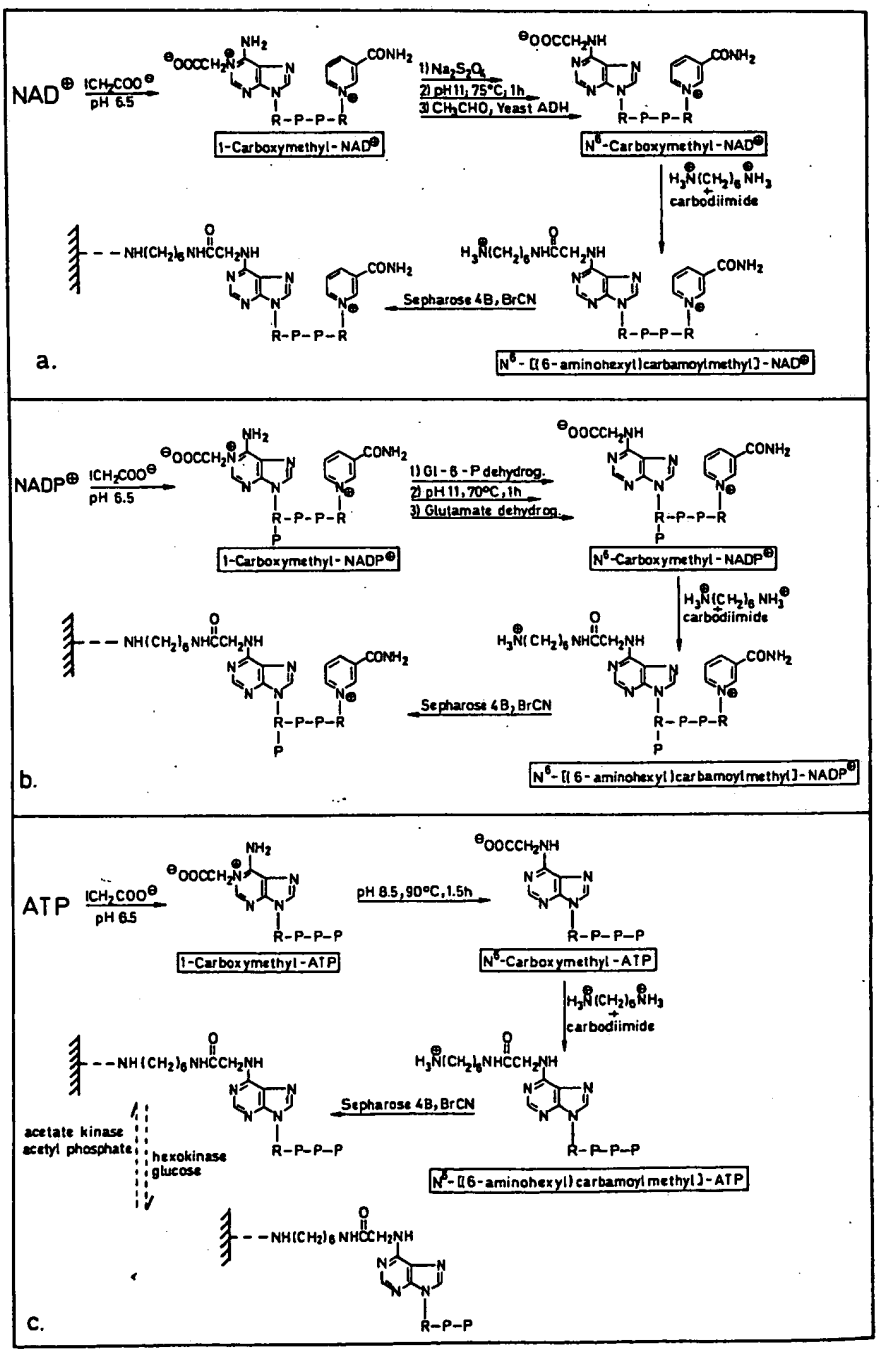
### Principle

The procedures given below follow the same general outline and are summarized in Fig. 1a–c: (a) alkylation with iodoacetic acid to give 1-carboxymethyl nucleotides; (b) rearrangement to the $N^6$-carboxymethyl nucleotides; (c) condensation with 1,6-diaminohexane to give $N^6$-[(6-aminohexyl)carbamoylmethyl] nucleotides.

In the alkylation step a 3- to 10-fold excess of iodoacetic acid is employed. The excess is necessary to ensure complete conversion of the parent nucleotide, since in aqueous media a substantial amount of the alkylation agent is hydrolyzed to hydroxyacetic acid. It is also beneficial to use the smallest reaction volume conveniently possible to enhance the rate of the alkylation reaction.

[1] This series Vol. 34 (1974).

JOURNAL NAME- Methods Enzymol VOL. 44 1976 PP. 859-87


NAD⊕
ICH2COO⊖ pH 6.5
1) Na2S2O4
2) pH 11, 75°C, 1h
3) CH3CHO, Yeast ADH
1-Carboxymethyl-NAD⊕
N6-Carboxymethyl-NAD⊕
carbodiimide
Sepharose 4B, BrCN
N6-[(6-aminohexyl)carbamoylmethyl]-NAD⊕
a.
NADP⊕
ICH2COO⊖ pH 6.5
1) Gl-6-P dehydrog.
2) pH 11, 70°C, 1h
3) Glutamate dehydrog.
1-Carboxymethyl-NADP⊕
N6-Carboxymethyl-NADP⊕
carbodiimide
Sepharose 4B, BrCN
N6-[(6-aminohexyl)carbamoylmethyl]-NADP⊕
b.
ATP
ICH2COO⊖ pH 6.5
pH 8.5, 90°C, 1.5h
1-Carboxymethyl-ATP
N6-Carboxymethyl-ATP
carbodiimide
Sepharose 4B, BrCN
acetate kinase
acetyl phosphate
hexokinase
glucose
N6-[(6-aminohexyl)carbamoylmethyl]-ATP
c.

By-products are virtually absent until the alkylation has reached a level of about 80%. If higher conversions are attempted, significant amounts of nucleotide by-products start to accumulate. The alkylation reaction should thus be terminated when a conversion of about 90% has been reached in order to obtain a maximum yield of 1-carboxymethyl nucleotide.

No attempt has been made to optimize the alkylation conditions for each of the nucleotides described, although the following may serve as a general rule of thumb for the various nucleotides: 5 days in the dark at room temperature and adjustment of pH once a day with 2 *M* LiOH. (Recent data obtained with AMP and $NAD^+$ suggest that on raising the temperature to 40°, the time required for alkylation can be reduced to 1 day provided pH adjustments are made more frequently.)

The 1-carboxymethyl nucleotides are intrinsically unstable, especially in alkaline media at elevated temperatures, and rearrange by a Dimroth mechanism to $N^6$-substituted nucleotides.[2] The 1-carboxymethyl derivatives of ADP and ATP are thus easily converted to $N^6$ derivatives merely by heating at 90°, pH 8.5, for 1.5 hr. These conditions are, somewhat surprisingly, not accompanied by hydrolytic release of phosphate or pyrophosphate. The 1-carboxymethyl derivatives of $NAD^+$ or $NADP^+$, on the other hand, cannot be satisfactorily rearranged in this direct fashion owing to their marked lability in alkali. However, reduction, either enzymically or with dithionite, yields the alkali-stable reduced nucleotide, which is rearranged subsequently by heating in alkali. The resulting $N^6$-carboxymethyl-NAD(P)H is then reoxidized enzymically. This reoxidation is necessary to permit purification by ion-exchange chromatography in acid media, which would otherwise destroy the extremely acid-labile reduced analogs. The enzymic reoxidation is preferable to other oxidation methods, since it is highly selective and will oxidize only those nucleotides that are correctly substituted and have the proper configuration. The beta form of $N^6$-carboxymethyl NAD(P)H is thus oxidized whereas the corresponding alpha form, which may be formed as an undesirable by-product during the rearrangement, will re-

FIG. 1a–c. Synthesis of the 1-carboxymethyl and $N^6$-[(6-aminohexyl)carbamoylmethyl] derivatives of $NAD^+$ (a), $NADP^+$ (b), and ATP (c). Several types of binding are reported for molecules bound to cyanogen bromide-activated gels. The linkage is indicated with a dashed line. R = ribose, P = phosphate, ADH = alcohol dehydrogenase, Gl-6-P dehydrog. = glucose-6-phosphate dehydrogenase.

In Fig. 1c is also given the enzymic interconversion of ATP ↔ ADP obtained on solid phase.

[2] M. H. Wilson and J. A. McCloskey, *J. Org. Chem.* 38, 2247 (1973).

main reduced. Those nucleotide molecules that remain reduced are rapidly destroyed in the subsequent acid media and are converted into compounds that are easily separated from the desired nucleotide by ion-exchange chromatography.

The $N^6$-carboxymethyl adenine nucleotides may be bound covalently to various matrices, or, alternatively, condensation of the $N^6$-carboxymethyl nucleotides with 1,6-diaminohexane generates $N^6$-[(6-aminohexyl)carbamoylmethyl] nucleotides bearing a terminal amino group suitable for direct attachment to supports. The latter analogs have been applied primarily by the authors. The condensation is efficiently promoted by water-soluble carbodiimides and, provided the diamine is present in 10-fold or higher excess, negligible formation of bis-nucleotides occurs.

A strongly red by-product is formed in small amounts, but may be conveniently removed by ethanol precipitation or absorption on an ion exchanger.

## Examples

### *$N^6$-Carboxymethyl-NAD$^+$*

The synthesis described below is essentially identical to a previously published procedure[3,4] (Fig. 1a).

$NAD^+$ (10 g, 13.6 mmoles Sigma grade AA) is added to an aqueous solution containing 30 g of iodoacetic acid (160 mmoles) neutralized with 2 *M* LiOH, and the pH of the mixture is readjusted to 6.5. The solution (total volume 135 ml) is kept in the dark at room temperature and the pH is adjusted to 6.5 with 2 *M* LiOH once every day. The alkylation is followed by thin-layer chromatography and, when the conversion of $NAD^+$ is approximately 90% (after 6–7 days), the reaction is terminated. The pH is lowered to 3.0 with 6 *M* HCl, two volumes of ethanol are added, and the solution is poured into 1.5 liters of vigorously stirred ethanol (0°). The precipitated crude 1-carboxylmethyl-$NAD^+$ is filtered off and washed with ethanol and ether and dried in a vacuum. Approximately 12 g of a faintly pink powder is obtained showing a purity with respect to nucleotide of about 90%. The crude 1-carboxymethyl-$NAD^+$ is dissolved in 300 ml of 2% $NaHCO_3$, the pH is adjusted to 8.5, and the solution is deaerated with 95% $N_2$–5% $CO_2$. The reducing agent sodium dithionite (5 g) is added, and, after 2 hr at room temperature in the dark,

[3] M. Lindberg, P. O. Larsson, and K. Mosbach, *Eur. J. Biochem.* 40, 187 (1973).
[4] K. Mosbach, this series Vol. 34, p. 233 (1974).

the reduction is terminated by oxygenation for 10 min followed by a brief treatment with nitrogen gas.

The reduced compound is subsequently rearranged to $N^6$-carboxymethyl-NADH by adjusting the pH of the solution to 11.5 with NaOH and heating at 75° for 1 hr.

Finally an enzymic reoxidation step is undertaken at room temperature by adding the following: 20 ml of 2 *M* Tris, 5 ml of redistilled acetaldehyde, 3 *M* HCl to a final pH of 7.5, and 5 mg of yeast alcohol dehydrogenase (2500 U). The oxidation is followed spectrophotometrically at 340 nm; it is considered complete when no further decrease of absorbance occurs (about 30 min). The solution is acidified with HCl to pH 3.5, and 1 volume of ethanol is added. The solution is then poured into 10 volumes of vigorously stirred ethanol, and the resulting suspension is kept in the refrigerator overnight. The crude $N^6$-carboxymethyl-NAD$^+$ is filtered off, washed with ethanol and ether, and dried. The product is purified by ion-exchange chromatography on Dowex AG 1X-2 (200–400 mesh, chloride). The column (2.5 × 75 cm) is loaded with a solution of the crude substance (pH 8) and then washed with water (500 ml) and 0.005 *M* $CaCl_2$, pH 2.7, until the pH of the effluent is about 2.8 (2.5 liters). A linear gradient, 0.005 *M* $CaCl_2$, pH 2.7, to 0.05 *M* $CaCl_2$, pH 2.0 (total volume 8 liters), is applied. The effluent between approximately 3.3 liters and 5.2 liters contains the desired compound. The eluate is neutralized with $Ca(OH)_2$ and concentrated on a rotary evaporator. Precipitation with ethanol as above gives pure $N^6$-carboxylmethyl-NAD$^+$ in a yield of 40–45% (5.6 g).

### $N^6$-[(6-Aminohexyl)carbamoylmethyl]-NAD$^+$

$N^6$-Carboxymethyl-NAD$^+$ (4.5 g, 5 mmoles) is dissolved in 60 ml of 2 *M* 1,6-diaminohexane dihydrochloride, and the solution is kept in an ice-bath. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (1.4 g, 7.5 mmoles) dissolved in 5 ml water is added, and the pH is kept at 4.8 by adding 1 *M* HCl or 1 *M* LiOH. After 10 min, when the reaction is slowing down, the ice-bath is removed and the condensation is allowed to proceed for a further 60 min at room temperature. The pH is then increased to 6.5, and 1 volume of 0.5 *M* LiCl in ethanol is added; the resulting red precipitate is filtered off and discarded. The supernatant is slowly poured into a 10-fold volume of vigorously stirred ethanol (0°C), and the precipitated $N^6$-[(6-aminohexyl)carbamoylmethyl]-NAD$^+$ is collected. The almost pure compound is dissolved in 75 ml of water (pH 8) and the solution is passed through a column containing 5 g of lithium-charged Dowex 50 W-X4. The effluent is adjusted to pH 6 with HCl and passed

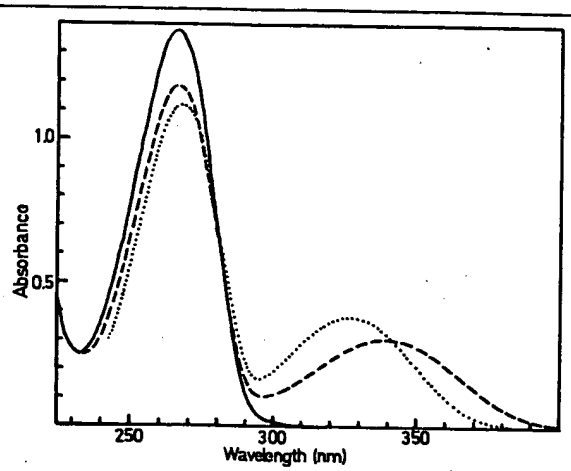



FIG. 2. Ultraviolet spectra of dextran-bound $N^6$-[(6-aminohexyl)carbamoylmethyl]-NAD$^+$ in 0.10 *M* Tris-HCl buffer, pH 7.5 (——), of the reduced form obtained after reduction for 15 min with yeast ADH (25 μg/ml) in 0.33 *M* ethanol and 0.10 *M* glycine-NaOH buffer, pH 9.5 (---), and of the KCN complex in 1.0 *M* KCN (· · · ·). The reference cells contained the same ingredients as the sample cells except that dextran-NAD(H) was replaced by blank dextran. The spectra of the corresponding free NAD$^+$-derivatives were identical with the exception of the enzymically reduced NAD$^+$ analog, which in its unbound state showed a higher ratio $A_{340nm}/A_{266nm}$ due to complete reduction.

through a column packed with 5 g of chloride-charged Dowex 1-X4. The final effluent is adjusted to 6.8 and concentrated to about 25 ml. Pure $N^6$-[(6-aminohexyl)carbamoylmethyl]-NAD$^+$ is obtained by ethanol precipitation carried out as above. The yield is about 80% (3.3 g), the overall yield from NAD$^+$ being approximately 35%. Based on phosphate determinations and UV measurements at 266 nm in Tris-HCl, pH 7.5, the molar absorption coefficient is determined as $\epsilon_{266\,nm}^{pH\,7.5} = 21{,}700\ M^{-1}\ cm^{-1}$ (Fig. 2).

### *$N^6$-Carboxymethyl-NADP$^+$*

The synthesis described below is essentially identical to a previously published procedure[5] (Fig. 1b).

Other methods of synthesis based on the condensation of nicotinamide mononucleotide with an appropriate adenosine-2′,5′-bisphosphate analog were considered inappropriate since cumbersome separation problems would be anticipated.[6,7] NADP$^+$ (1.0 g, 1.3 mmoles) is added to an aqueous solution of iodoacetic acid (1.0 g, 5.4 mmoles) neutralized with standard 2 *M* LiOH. The pH is readjusted to 6.5, and the solution (total

[5] C. R. Lowe and K. Mosbach, *Eur. J. Biochem.* 49, 511 (1974).
[6] D. B. Craven, M. J. Harvey, and P. D. G. Dean, *FEBS Lett.* 38, 320 (1974).
[7] N. A. Hughes, G. W. Kenner, and A. Todd, *J. Chem. Soc.* 3733 (1957).

volume 10.0 ml) is kept in the d[redacted]at 26°–27°. The pH is checked daily and readjusted to 6.5 with 2 *M* LiOH when necessary. The progress of the reaction may be followed by thin-layer chromatography on poly-(ethyleneimine) cellulose using 0.5 *M* LiCl as eluent. After 10 days, most of the $NADP^+$ is converted to $N^1$-carboxymethyl-$NADP^+$ whence the reaction mixture is acidified to pH 3.0 with 3 *M* HCl, cooled to 0°, and slowly added to a 10-fold excess of ethanol at —20°. The flocculent pale pink precipitate is collected, washed extensively with cold ethanol and ether, and finally air-dried.

$N^1$-Carboxymethyl-$NADP^+$ is intrinsically unstable and rearranges under alkaline conditions and elevated temperatures to the $N^6$-substituted form. To prevent hydrolysis during these steps the nucleotide is enzymically converted to the alkali-stable reduced form. Glucose 6-phosphate (612.3 mg, 2 mmoles), $N^1$-carboxymethyl-$NADP^+$ (765.0 mg, 1 mmole), and yeast glucose-6-phosphate dehydrogenase (250 units) are added to 30 ml 0.1 *M* Tris-HCl buffer pH 7.4 containing 5 m*M* Mg $Cl_2$ and incubated for 1.5 hr at 25°. The progress of the reduction is followed by the increase in absorbance at 340 nm and is essentially complete after 30 min. The rearrangement is effected by adjusting the pH of the solution to 11.0 with 1 *M* NaOH and heating for 1 hr at 70°. The solution is cooled to 25°, and the pH is readjusted to 7.4. Ammonium acetate (246.6 mg, 3.2 mmoles), 2-oxoglutarate (116.9 mg, 0.8 mmole), EDTA (3 mg), and beef liver glutamate dehydrogenase (100 units) are added, and the reoxidation allowed to proceed for 1.5 hr at 25°. The reaction is monitored by the decrease in absorbance at 340 nm; on completion, the solution is cooled in ice, poured slowly into cold ethanol (—20°), and allowed to stand overnight at —20°. An oily precipitate forms and sediments overnight; it is collected, washed with cold ethanol and ether, and finally air-dried. The crude powder (1.3 g) is dissolved in 400 ml of distilled water followed by adjustment of the pH to 7.5 with 0.1 *M* NaOH. The solution is applied to a Dowex 1-X2 column ($Cl^-$; 1.5 × 25 cm) that has previously been equilibrated with distilled water. The column is washed with water (30 ml) and then with 1 m*M* $CaCl_2$, pH 3.0, until the effluent no longer contains UV-absorbing material and the pH has attained a value of 3.0. The nucleotides are eluted with a linear gradient of $CaCl_2$, 1 m*M* $CaCl_2$, pH 3.0, to 100 m*M* $CaCl_2$, pH 2.0, 800 ml total volume, at a flow rate of 70–80 ml/hour. Fractions (5 ml) are collected, and those in the two major peaks containing nucleotide are pooled independently. The pooled peak fractions are adjusted to pH 7.5 with 2 *M* NaOH, diluted 4-fold with distilled water and concentrated on a small Dowex 1-X2 column (Cl; 0.7 × 3 cm) equilibrated with distilled water. Nucleotide is eluted with 100 m*M* $CaCl_2$ pH 2.0 and the concentrated pale-straw-colored solution is desalted on a column of Sephadex G-10 (3 × 41 cm) equilibrated with water. Elution is effected with water at a

flow rate of 3.2 ml/minute, and the desalted fractions containing UV-absorbing material are lyophilized. The two major peaks of nucleotide eluted from the first Dowex-1 column are thus obtained as white salt-free lyophilizates homogeneous by thin-layer chromatography in several systems and shown to be $N^1$-carboxymethyl-NADP$^+$ and $N^6$-carboxymethyl-NADP$^+$, respectively. The yield of the latter varies from preparation to preparation, but based on phosphate determinations and UV measurements at 265 nm in Tris-HCl buffer pH 7.5, the molar absorption coefficient is 21,800 $M^{-1} \times cm^{-1}$, and the yield is in the range 15–25% (100–200 mg). The purified $N^1$-carboxymethyl-NADP$^+$ generated at this stage can be recycled through the rearrangement to produce more $N^6$-carboxymethyl-NADP$^+$.

### *$N^6$-[(6-Aminohexyl)carbamoylmethyl]-NADP$^+$*

$N^6$-Carboxymethyl-NADP$^+$ (37.5 mg, 45.6 $\mu$moles) is dissolved in 25 ml of an 1 *M* aqueous solution of 1,6-diaminohexane dihydrochloride, and the pH is adjusted to 4.7. The water-soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (67.9 mg, 350 $\mu$moles) is added slowly in portions, and the pH is adjusted to 4.7 with 0.1 *M* HCl after each addition. The reaction is allowed to proceed for 20 hr at 21°–22° with periodic readjustments of the pH to 4.7, and then freed from carbodiimide and 1,6-diaminohexane by passage through a Sephadex G-10 column (3 × 41 cm) equilibrated with distilled water. The pooled nucleotide-containing fractions are applied directly to a Dowex 1-X2 column ($Cl^-$; 3.1 × 7.7 cm) and subsequently eluted with a linear gradient of $CaCl_2$, 1 m*M* $CaCl_2$, pH 3.0, to 100 m*M* $CaCl_2$, pH 2.0, 20 ml total volume. Fractions (1.5 ml) are collected, and those containing $N^6$-[(6-aminohexyl)carbamoylmethyl]-NADP$^+$ pooled, desalted by passage through a Sephadex G-10 column as above, and finally lyophilized. The product is essentially salt-free and homogeneous in several thin-layer chromatography systems. Under the above conditions, 80% of the $N^6$-carboxymethyl-NADP$^+$ is converted to $N^6$-[(6-aminohexyl)carbamoylmethyl]-NADP$^+$ based on phosphate analysis and UV data ($\epsilon_{265\,nm}^{pH\,7.5}$ = 21,700 $M^{-1} \times cm^{-1}$). Excess carbodiimide should be avoided because some nucleotide 2′,3′-cyclic phosphate might be formed which could contaminate the 3′-isomer of the NADP$^+$-analog.

### *$N^6$-Carboxymethyl-ATP*

The synthesis described below is essentially identical to a previously published procedure[8] (Fig. 1c).

[8] M. Lindberg and K. Mosbach, *Eur. J. Biochem.* 53, 481 (1975).

ATP (5 g, 8.3 mmoles) is added to an aqueous solution of iodoacetic acid (15 g, 80 mmoles) neutralized with 2 *M* LiOH, and the pH is readjusted to 6.5. The solution (total volume 60 ml) is kept in the dark at 30°, and the pH is kept constant by adjustment with 2 *M* LiOH. After 5 days most of the ATP is converted to 1-carboxymethyl-ATP as indicated by thin-layer chromatography. The product is precipitated by slowly adding 8 volumes of chilled ethanol (—20°) to the vigorously stirred reaction mixture; the precipitate is filtered off and washed with ethanol. To effect rearrangement to $N^6$-carboxymethyl-ATP, the precipitate is dissolved in 100 ml of water, the pH is adjusted to 8.5, and the solution is heated at 90° for 1.5 hr. The pH value is checked intermittently and adjusted when necessary with 1 *M* LiOH.

The solution containing the rearranged compound is cooled, adjusted to pH 2.75 with 1 *M* HCl, and applied to a Dowex 1-X2 column (200–400 mesh, $Cl^-$, 4 × 30 cm). The column is washed with 0.3 *M* LiCl, pH 2.75, until the effluent contains no ultraviolet-absorbing material. A linear LiCl gradient is then applied, 0.3 *M* LiCl, pH 2.75, to 0.5 *M* LiCl, pH 2.0 (total volume, 4 liters). The pooled fractions comprising the main peak are neutralized with 1 *M* LiOH and concentrated on a rotary evaporator to a final volume of 80 ml. The yield is approximately 50% based on a molar absorption coefficient of $\epsilon_{267\,nm}^{pH\,7.5} = 17{,}300\ M^{-1}\ cm^{-1}$ determined for a similar analog, $N^6$-(6-aminohexyl)-AMP.[9]

### *$N^6$-[(6-Aminohexyl)carbamoylmethyl]-ATP*

To produce the $N^6$-[(6-aminohexyl)carbamoylmethyl]-ATP, 80 ml of a 1 *M* 1,6-diaminohexane dihydrochloride solution are added to the above solution. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (1.5 g, 8 mmoles) dissolved in 5 ml of water is added dropwise, and the pH is maintained at 4.7 by adding 0.5 *M* HCl. After 1 hr at room temperature, thin-layer chromatography indicates practically total conversion of the $N^6$-carboxymethyl-ATP. The product is collected by precipitation with 8 volumes of chilled ethanol–acetone (1:1) and is subsequently applied at pH 5 to a Dowex 1-X2 column (200–400 mesh, $Cl^-$, 4 × 30 cm). The column is washed with 100 ml of 0.05 *M* LiCl, pH 5, and then eluted with a linear LiCl gradient, 0.05 *M* LiCl, pH 5, to 0.35 *M* LiCl, pH 2 (total volume, 4 liters). The pooled fractions of the main peak are neutralized and concentrated on a rotary evaporator to a final volume of 50 ml. The product is precipitated with 10 volumes of cold acetone–ethanol mixture (1:1) and is made essentially salt-free by repeated

[9] H. Guilford, P. O. Larsson, and K. Mosbach, *Chem. Scripta* 2, 165 (1972).

washings with the above mixture. Finally, $N^6$-[(6-aminohexyl)carbamoylmethyl]-ATP is dissolved in a small amount of water and precipitated by adding 8 volumes of chilled ethanol. The yield is 75% based on the amount of $N^6$-carboxymethyl-ATP.

### *$N^6$-[(6-Aminohexyl)carbamoylmethyl]-ADP*

$N^6$-[(6-Aminohexyl)carbamoylmethyl]-ADP is prepared according to the above procedure using $N^6$-carboxymethyl-ADP as an intermediate. The overall yield is the same, i.e., about 40%.

### *$N^6$-[(6-Aminohexyl)carbamoylmethyl]-AMP*

$N^6$-[(6-Aminohexyl)carbamoylmethyl]-AMP is prepared according to the above procedure using $N^6$-carboxymethyl-AMP as an intermediate in an overall yield of 45%.

## Characterization

The method of synthesis and the properties of the intermediate and products all support the structures assigned to the $N^6$-carboxymethyl and $N^6$-[(6-aminohexyl)carbamoylmethyl] derivatives of ADP, ATP, $NAD^+$, and $NADP^+$. The 1-alkylated nucleotides thus have absorption maximum at 259 nm and a broad shoulder with inflection point at 290 nm. Rearrangement is accompanied by a characteristic shift from 260 nm of the absorption maximum to around 266 nm and disappearance of the shoulder at 290 nm. These spectral properties are in accord with the known properties of analogous hydroxyethyl-substituted[10] and benzyl-substituted[11] nucleotides.

Chemical and enzymic reduction of the $NAD^+$ and $NADP^+$ analogs gives the expected peak at 340 nm, and incubation with 1 *M* KCN gives a peak at 325 nm typical of quaternary nicotinamide-cyanide addition compounds (Fig. 2).

Treatment of the $NAD^+$-analogs with nucleotide pyrophosphatase yields, besides NMN, fragments identical with $N^6$-carboxymethyl-AMP or $N^6$-[(6-aminohexyl)carbamoylmethyl]-AMP, both prepared by a different method of synthesis (from 6-chloropurine riboside phosphate).[9]

The various nucleotides show $R_f$ values in good agreement with those expected from their net charges and are summarized in Table I.

The ADP, ATP, and $NADP^+$ derivatives are readily hydrolyzed by

[10] H. G. Windmueller and N. O. Kaplan, *J. Biol. Chem.* 236, 2716 (1961).
[11] J. W. Jones and R. K. Robins, *J. Am. Chem. Soc.* 85, 193 (1963).

TABLE I
THIN-LAYER CHROMATOGRAPHY

| Compound | $R_f$ values in chromatography systems[a] | | | | | | |
|---|---|---|---|---|---|---|---|
| | Silica | Cellulose | | Polyethyleneimine cellulose | | | |
| | A | A | B | C | D | E | F |
| AMP | — | 0.46 | 0.25 | — | — | — | 0.60 |
| $N^6$-R″-AMP[b] | — | 0.67 | 0.42 | — | — | — | 0.89 |
| ADP | — | 0.41 | 0.30 | — | — | — | 0.30 |
| $N^6$-R″-ADP | — | 0.61 | 0.47 | — | — | — | 0.78 |
| ATP | — | 0.35 | 0.37 | — | — | — | 0.13 |
| $N^6$-R″-ATP | — | 0.56 | 0.56 | — | — | — | 0.51 |
| $NAD^+$ | 0.44 | 0.45 | — | 0.80 | 0.51 | 0.74 | — |
| $N^6$-R′-$NAD^+$[c] | 0.22 | 0.20 | — | 0.17 | 0.17 | 0.81 | — |
| $N^6$-R″-$NAD^+$ | 0.44 | 0.55 | — | >0.95 | >0.95 | >0.95 | — |
| $NADP^+$ | 0.31 | 0.32 | — | 0.07 | 0.02 | 0.30 | — |
| $N^6$-R′-$NADP^+$ | 0.14 | 0.16 | — | 0.01 | 0.01 | 0.20 | — |
| $N^6$-R″-$NADP^+$ | 0.34 | 0.48 | — | 0.59 | 0.69 | >0.95 | — |

[a] Solvent systems: A, isobutyric acid—1 *M* aqueous ammonia (5:3, v/v), solvent saturated with disodium EDTA; B, 0.1 *M* potassium phosphate, pH 6.8–ammonium sulfate–1-propanol (100:60:2, v/w/v); C, 3 *M* acetic acid; D, 0.1 *M* LiCl; E, 0.5 *M* LiCl; F, 1 *M* LiCl.
[b] R″ = (6-aminohexyl)carbamoylmethyl.
[c] R′ = carboxymethyl.

alkaline phosphatase, proving that no alkylation of the phosphate entities by iodoacetic acid has occurred and the carbodiimide-promoted attachment of the 1,6-diaminohexane spacer has not resulted in phosphoramidate formation. This is further evidenced by the fact that the $N^6$-[(6-aminohexyl)carbamoylmethyl] nucleotides contain just one aliphatic amino group (trinitrobenzene sulfonic acid test) per molecule. Furthermore, the properties of the analogs as active cofactors and as affinity chromatography ligands with several dehydrogenases and kinases are also in agreement with their assigned structures.

### Binding to Matrices

For affinity chromatography purposes, cofactor and cofactor analogs have been almost exclusively attached to water-insoluble supports, such as Sepharose and porous glass, to allow use of convenient column techniques. Important requirements of the matrix are sufficient porosity to allow unimpeded passage of macromolecules and lack of nonspecific sites

that might adversely interfere with the separation processes. These properties are also necessary for carriers of active cofactors, but the need for fast and efficient interaction between polymer-bound cofactor and enzyme becomes a dominant feature. In this respect the solid supports are far from ideal. The severely restricted mobility imposed on the bound cofactor will slow down its interaction with the enzyme. Experiments with Sepharose-bound $N^6$-[(6-aminohexyl)carbamoylmethyl]-$NAD^+$ as cofactor for yeast alcohol dehydrogenase and lactate dehydrogenase[12] clearly show that the efficiency of the bound cofactor is very low compared to corresponding free systems. Even when a very mild activation with CNBr of the polymer was undertaken to minimize cross-linking of the gel, only about 30% of the bound cofactor molecules could be enzymically reduced. The remaining 70% of the analogs are obviously bound to the matrix in such a way that proper interaction with the enzyme is prohibited owing to shielding effects imposed by the matrix backbone.

In contrast, with soluble carriers like dextran, about 80% of the cofactor was enzymically reducible indicating a less sterically hindered enzyme–coenzyme interaction (Fig. 2).

The nucleotides described above are provided either with a terminal carboxyl function or an amino function. The well-known coupling procedures involving, for instance, carbodiimides or cyanogen bromide (CNBr) are thus suitable for binding the analogs to soluble or insoluble polymers. The carboxymethyl derivatives may be coupled to carriers functionalized with extension arms bearing terminal amino groups using carbodiimide. This approach suffers from the inherent limitation that the resulting cofactor polymer will contain an excess of unsubstituted extension arms after coupling that might seriously impede proper coenzyme–enzyme interaction. This has been pointed out in affinity chromatography on several occasions, but in our experience it is also relevant when designing immobilized active cofactors. It was thus found that $N^6$-carboxymethyl-$NAD^+$ when coupled to 1,6-diaminohexane-substituted soluble dextran showed a very erratic behavior as coenzyme for yeast alcohol dehydrogenase. Several experiments, including tests with 1,6-diaminohexane-substituted dextran together with the free $NAD^+$ or $NAD^+$-analog, led to the conclusion that the enzyme gradually becomes quite tightly bound to the polymer and in such a manner that catalytic activity is almost entirely abolished. Furthermore, soluble polyethyleneimine, which also has an abundance of charged groups, showed a similarly disappointing behavior as cofactor carrier in our hands with yeast alcohol dehydrogenase. The liver enzyme, however, was insensitive to the excess of charged groups on the carrier, and it may well be that other enzymes and

[12] P. O. Larsson and K. Mosbach, *FEBS Lett.* 46, 119 (1974).

supports will also yield useful preparations with the short $N^6$-carboxymethyl adenine nucleotides described. At present we suggest the use of a preformed coenzyme-spacer entity of the type $N^6$-[(6-aminohexyl)carbamoylmethyl]-$NAD^+$, and then attach it, for instance, to CNBr-activated polymers (concerning matrix-binding, see further below). Such preparations will not carry unsubstituted spacers.

Some precautions must be observed on coupling analogs to soluble dextran if preparations of high efficiency are desired. Activation with a high concentration of cyanogen bromide results in extensive cross-linking of the dextran, which might lead to insoluble derivatives, especially if the activated dextran is freed from excess cyanogen bromide by precipitation with organic solvents. In contrast, low CNBr concentrations cause little cross-linking, and, since all cyanogen bromide is rapidly consumed, it is not necessary to purify the activated dextran prior to cofactor addition. The coupling yield is in the range of 30–50%, and the resulting products have excellent cofactor properties and are enzymically reducible to the extent of about 80%. If higher coupling yields are attempted by increasing the degree of CNBr activation, a substantial portion of the cofactor cannot interact with the enzymes, probably owing to shielding effects from the cross-linked polymer.

One other problem associated with CNBr activation has recently become apparent. Activation of Sepharose with CNBr leads to attachment of the ligand through predominantly N-substituted isourea bonds. These linkages are not completely stable, and the conjugates exhibit a small but constant "leakage" from the solid matrix. This leakage is particularly acute at high temperatures, at extreme pH values, and if nucleophiles are present in the surrounding media (under normal pH and temperature conditions, however, and, e.g., when applied in phosphate buffer, "leakage problems" can be disregarded). The problem may be circumvented by using alternative methods of coupling of the ligand to the matrix (e.g., to epoxy-gels) and/or other solid support materials. Full details of these alternative procedures are given in this volume.

A few examples of coupling the nucleotides to the carriers dextran and Sepharose are given below with, for the above reasons, greater emphasis placed on soluble matrices. The procedures, however, are likely to be generally applicable to all the $N^6$-[(6-aminohexyl)carbamoylmethyl] nucleotides.

### *Preparation of Dextran-$NAD^+$ and -$NADP^+$*

A more detailed procedure is given below for dextran-$NAD^+$ essentially following a published procedure.[12] A shorter description on dextran-

NADP+ has also been published previously.[5] It is likely that the somewhat different procedure given for NAD+ is also applicable for NADP+.

*Dextran-NAD+.* Dextran T 40 (Pharmacia, Uppsala, Sweden) (5.0 g) is dissolved in 50 ml of water (20°); a solution of 0.25 g of CNBr in 5 ml of water is added, and the pH is maintained at 10.8 by continuous addition of 1 *M* NaOH. After approximately 5 min all CNBr is consumed (no further consumption of NaOH and no cyanide smell), and the activation is judged complete. The pH is lowered to 8.5 with 0.1 *M* HCl, and 0.90 mmoles of $N^6$-[(6-aminohexyl)carbamoylmethyl]-NAD+ dissolved in 5 ml of water is added. The coupling is allowed to proceed at pH 8.5 for 12 hr at room temperature, whence any residual active groups are quenched by treatment with 0.2 *M* ethanolamine-HCl buffer, pH 8.0, for 1 hr at room temperature. The reaction mixture is diluted to 500 ml with 0.1 *M* LiCl, adjusted to pH 6.8 with HCl, and applied to a Sephadex G-50 column (5 × 85 cm). The dilution lowers the viscosity enough to prevent anomalous gel filtration behavior. Elution is performed with 0.1 *M* LiCl to suppress any ion pair formation between polymer and unbound nucleotide and the effluent is collected as 20-ml fractions. Fractions 25–50 contain dextran-NAD+, and fractions 65–100 uncoupled analog. The dextran derivative is concentrated on a rotary evaporator (30°) to approximately 100 ml and then pipetted into 1.5 liters of vigorously stirred ethanol. The precipitate is filtered off, washed with ethanol and ether, and dried in a vacuum. The yield is 5.1 g of a white powder.

Ultraviolet measurements (see also Fig. 2) indicate a nucleotide content of 65 μmoles per gram of dry dextran and a coupling yield of about 40% (assuming a molar absorption coefficient of 21,700 $M^{-1} \cdot cm^{-1}$). The uncoupled nucleotide may be recovered after concentration of fractions 65–100 to approximately 50 ml and precipitation with 500 ml of ethanol. A "blank" dextran is prepared by following the same procedure. The nucleotide in this case is replaced by, for example, 0.90 mmole of butylamine.

*Dextran-NADP+.* Dextran-bound NADP+ is prepared by dissolving Dextran T 40 (0.5 g) in 10 ml of distilled water and activating at pH 11.0 ± 0.2 by the stepwise addition of 25 mg of CNBr in 1 ml of water and continuous titration with 0.5 *M* NaOH at 25°. On completion of the reaction, 1 ml of the activated dextran solution is added to approximately 10 μmoles of $N^6$-[(6-aminohexyl)carbamoylmethyl]-NADP+ in 50 μl of 0.1 *M* $NaHCO_3$, pH 8.5, and allowed to stand overnight at 4°. The solution is applied to a column of Sephadex G-50 (3 × 40 cm) equilibrated with water and eluted at a flow rate of 150 ml/hr. The fractions (2.0 ml) eluted immediately after the void volume and showing absorbance at 265 nm are free of uncoupled NADP+ analog and are used in all subse-

quent studies. The resulting dextran contains up to 85 μmoles of $NADP^+$ analog per gram of dry dextran when based on absorbance measurements at 265 nm.

### *Preparation of Sepharose-$NAD^+$, -$NADP^+$, and -ATP*

Coupling of the $NAD^+$ analog to Sepharose has been described previously[3,4] and is essentially identical to that described below in more detail for $NADP^+$. Coupling of the ATP analog is also equivalent and has been described.[8]

*Sepharose-$NADP^+$.* Sepharose 4B is activated by the CNBr method using 30 mg of CNBr per milliliter of gel. The activated gel is thoroughly washed with ice cold 0.1 *M* $NaHCO_3$, pH 8.5, and 1 g is added to 20 μmoles of $N^6$-[(6-aminohexyl)carbamoylmethyl]-$NADP^+$ in 0.5 ml of the same buffer. Coupling is allowed to proceed for 20 hr at 0°–4°, whence the gel is exhaustively washed with 0.1 *M* $NaHCO_3$, pH 8.5, 2 *M* KCl, and distilled water. Under these conditions the amount of bound nucleotide is approximately 1.1 μmole per gram of wet gel or 27.5 μmoles per gram of dry gel according to UV measurements. The immobilized nucleotide is stable as a packed gel at 4° for several months.

## Coenzymic Activities of the Various $NAD^+$, $NADP^+$, and ATP Analogs Described

Of obvious importance for the utilization of immobilized coenzymes is the fact that the "coenzymic activity" of the analogs compared to the unmodified coenzymes is retained or not too severely diminished. Needless to say, this same rigid requirement does not necessarily apply when these cofactors are used as binding ligands in affinity chromatography. Substitution with a spacer group at the exocyclic $N^6$ group of the adenine moiety has been chosen in all the analogs described here, since this position appeared to be the most suitable as judged from X-ray crystallographic studies on $NAD^+$-dependent dehydrogenases and the good coenzymic activities reported from a similarly substituted hydroxyethyl $NAD^+$ analog.[10]

In Tables II, III, and IV the coenzymic activities of the $NAD^+$-, $NADP^+$-, and ATP-analogs with several enzymes are given relative to their parent nucleotides under identical conditions. A comparison of the kind given, combining $V_{max}$ and $K_m$, is of value, since it will provide information as to coenzymic activity under conditions rather similar to those prevailing in practical use. Alternatively the $k_{cat}$, $V_{max}$, and $K_m$ of the analogs could be given separately.

TABLE II
COENZYMIC ACTIVITY OF NAD+ ANALOGS

| Enzyme | Rate of reduction of[a] $N^6$-R′-NAD+[b] | $N^6$-R″-NAD+[c] | $N^6$-R″-NAD+-carrier |
|---|---|---|---|
| Alcohol dehydrogenase (liver) | 55 | 100 | — |
| Lactate dehydrogenase (beef heart) | 65 | 50 | — |
| Malate dehydrogenase | 75 | 75 | — |
| Alcohol dehydrogenase (yeast)[d,e] | — | 61 | 16 (Dextran T 10)<br>0.7 (Sepharose 4B) |

[a] The enzymic reduction of NAD+ and NAD+ analogs was measured by following the increase in absorbance at 340 nm[1]. All assays were performed at 24° in 0.1 *M* Tris-HCl buffer, pH 8.5, in a total volume of 1.0 ml. Incubation mixtures contained 100 μmoles of substrate and 0.50 μmole coenzyme or analog. Sufficient enzyme was added to obtain an initial reduction rate of 5–10 % of the coenzyme per minute. Rates are in relation to NAD+ = 100.

[b] R′ = carboxymethyl.

[c] R″ = (6-aminohexyl)carbamoylmethyl.

[d] Cofactor concentration = 0.1 m*M*.

[e] From P. O. Larsson and K. Mosbach, *FEBS Lett.* **46**, 119 (1974).

Because of the great variations of coenzymic activity found with the different NADP+ analogs, as shown in Table III, this aspect is discussed in somewhat more detail below.

The ability of the NADP+ analogs to function as coenzymes was examined by comparing the rates of reduction of the analogs with the unmodified rates for NADP+ in identical molar concentrations. Table IV shows the relative rates of reduction with several NADP+-dependent enzymes and with a typical NAD+-dependent enzyme. Most of the NADP+-dependent enzymes are active with both $N^1$- and $N^6$-substituted carboxymethyl analogs of NADP+, with some preference for the $N^1$-substituted derivatives. Altering the charge and bulk of the substituent on the adenine from $-CH_2COO^-$ to $-CH_2CONH(CH_2)_6NH_3^+$ significantly decreased the coenzymic activity of the analogs. These observations contrast with the coenzymic activities of correspondingly substituted derivatives of NAD+, where substantial coenzymic activity was obtained only with the $N^6$-substituted derivatives and where altering the charge and bulk of the substituent had little effect on the rate of reduction. L-Glutamate dehydrogenase utilizes both NAD+ and NADP+ as coenzyme and exhibits activity with both $N^1$- and $N^6$-carboxymethyl derivatives of NADP+ and, to a lesser extent, with the corresponding $N^6$-[(6-aminohexyl)carbamoylmethyl] analogs. Isocitrate dehydrogenase was totally

TABLE III
COENZYMIC ACTIVITY OF NADP$^+$ ANALOGS[a,b]

| Enzymes | NAD$^+$ | NADP$^+$ | $N^1$-R′-NADP$^+$[c] | $N^1$-R″-NADP$^+$[d] | $N^6$-R′-NADP$^+$[c] | $N^6$-R″-NADP$^+$[d] | $N^6$-R″-NADP$^+$-dextran[d] |
|---|---|---|---|---|---|---|---|
| Glucose-6-phosphate dehydrogenase | 0 | 100 | 145 | >5 | 65 | <5 | 35 |
| 6-Phosphogluconate dehydrogenase | 0 | 100 | 60 | >5 | 80 | 5 | 10 |
| L-Glutamate dehydrogenase | 130 | 100 | 50 | 10 | 35 | 10 | 15 |
| *threo*-D$_s$-Isocitrate dehydrogenase | 0 | 100 | 70 | 0 | 0 | 0 | 0 |
| Yeast alcohol dehydrogenase | 100 | 0 | 0 | 0 | 0 | 0 | 0 |

[a] From C. R. Lowe and K. Mosbach, *Eur. J. Biochem.* **49**, 511 (1974).

[b] The enzymic reduction of NADP$^+$, NAD$^+$, and the NADP$^+$ analogs was measured spectrophotometrically by following the increase in absorbance at 340 nm in 0.1 *M* Tris-HCl buffer, pH 7.5 (total volume 1 ml) at 25°. Standard assay procedures were used throughout except for beef liver glutamate dehydrogenase, which was assayed in the unfavorable direction using 10 μmoles of L-glutamate as substrate. Sufficient NADP$^+$ was added to give an absorbance of 1.00 at 260 nm, and enzyme was added to give a $\Delta A_{340}$/min of about 0.1. The same molar concentration of NADP$^+$ analog was achieved by suitably adjusting the absorbance in the cuvette. All values are quoted relative to NADP$^+$ (100) and represent the mean of three determinations.

[c] R′ = carboxymethyl.

[d] R″ = (6-aminohexyl)carbamoylmethyl.

TABLE IV
COENZYMIC ACTIVITY OF ATP ANALOGS

| Enzyme | Activity on ATP | $N^L$-R′-ATP[a] | $N^6$-R′-ATP[a] | $N^6$-R″-ATP[b] |
|---|---|---|---|---|
| Hexokinase[c,d] | 100 | 35 | 65 | 95 |
| Glycerokinase[e] | 100 | <5 | — | 20 |
| Phosphoglycerate kinase[f] | 100 | 0 | 0 | 0 |

[a] R′ = carboxymethyl.
[b] R″ = (6-aminohexyl)carbamoylmethyl.
[c] Standard assay procedures scaled down to 1 ml were used throughout. In each case where ATP was used, its concentration was 10 $K_m$ for the enzyme in question; the ATP analogs were tested at the same molar concentrations. All values are quoted relative to ATP (100) and represent the means of three determinations.
[d] Hexokinase activity was determined in a coupled assay with glucose-6-phosphate dehydrogenase; the production of NADPH was measured at 340 nm.
[e] Glycerokinase activity was determined in a coupled assay with glycerol-3-phosphate dehydrogenase-triosephosphate isomerase; the production of NADH was measured at 340 nm.
[f] Phosphoglycerate kinase was assayed in a coupled system with glyceraldehyde-3-phosphate dehydrogenase; the formation of $NAD^+$ was measured at 340 nm.

inactive with $N^6$-substituted derivatives. The $NAD^+$-dependent enzyme, alcohol dehydrogenase, was inactive with $NADP^+$ and all the analogs tested. Table III also lists the rate of reduction of dextran-bound $NADP^+$ relative to free unmodified $NADP^+$ for several enzymes. The higher rate of reduction of the dextran-bound $NADP^+$ compared to the free $N^6$-[(6-aminohexyl)carbamoylmethyl] analog may reflect the suppression *per se* of the positive charge on the terminal amino group of the spacer arm assembly or, alternatively, the inhibition of its potential intramolecular interaction with the negative charge on the 2′-phosphate. The dextran-bound $NADP^+$ analog is inactive with isocitrate dehydrogenase and alcohol dehydrogenase.

## Applications in Enzyme Technology and Analysis

The development of efficient enzyme-catalyzed processes is a prerequisite to the use of enzyme technology on a large scale. Many of these chemical processes will be catalyzed by enzymes that require the participation of readily dissociable coenzymes. Such coenzymes are often expensive, and thus their economical utilization would require methods both to retain them in the reaction mixture and to regenerate them. The immobilization of a coenzyme to a carrier macromolecule and its subse-

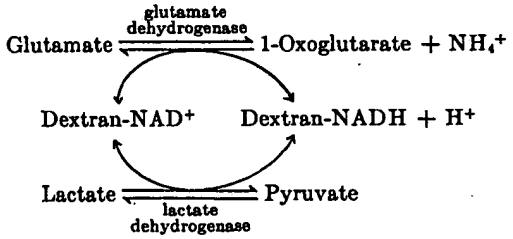
quent retention within a limiting membrane provide one solution to the problem.

Two examples where this approach has been applied are given below and utilize the dextran-bound $NAD^+$ analog, $N^6$-[(6-aminohexyl)carbamoylmethyl]-$NAD^+$.

## Enzyme Electrode

Immobilized coenzymes appear to be well suited to applications in enzyme electrodes (see chapter [41] in this volume). Enzymes and coenzymes can be coretained within a limiting membrane around, for instance, an ion-selective electrode and used to determine in a continuous or serial fashion metabolite concentrations under conditions that do not perturb the media to be analyzed. This will permit unrestricted monitoring of metabolite concentrations in physiological fluids *in vivo*, where it would normally not be possible to add a free coenzyme, and in addition the possible reuse of the immobilized coenzyme will make such analyses cheaper.

The coentrapment of coenzymically active dextran-bound $NAD^+$[13] together with soluble lactate dehydrogenase/glutamate dehydrogenase within an enzyme electrode has been used to determine glutamate concentrations. The presence of glutamate in an assay medium containing pyruvate generated $NH_4^+$ which was recorded by a $NH_4^+$-sensitive electrode (Scheme 1).




SCHEME 1. The generation of $NH_4^+$ by glutamate.

Alternatively the lactate dehydrogenase/glutamate dehydrogenase electrode can be used to determine pyruvate concentrations.

## Example

### *Materials and Methods*

The dialysis membranes were manufactured by the Union Carbide Corporation (Chicago, Illinois). They were treated before use by boiling

for 1 hr in 1 m*M* EDTA (sodium salt) (pH 7.0) and 1 hr in distilled water. The dextran-bound $NAD^+$, $N^6$-[(6-(aminohexyl)carbamoylmethyl]-$NAD^+$, contained 30 $\mu$moles of nucleotide per gram dry wt of dextran. The $NH_4^+$-sensitive glass electrode (Beckman 39137 cation-sensitive electrode) used in these assays responds to protons. Therefore all solutions used in determinations with this electrode were adjusted to pH 8.0 with Tris base at 25°. Since the electrode can also detect monovalent ions like $Na^+$ and $K^+$, all reagents used with the electrode were converted to their respective Tris salts. Sodium pyruvate was first converted to the free acid by passage through a column of Dowex 50 and then adjusted with Tris base to pH 8.0. ADP (sodium salt) was directly converted to the Tris salt by passage through Dowex 50 in the Tris form followed by adjustment to pH 8.0 as above.

The enzyme electrode was prepared by enclosing soluble glutamate dehydrogenase and rabbit muscle lactate dehydrogenase, and dextran-bound $NAD^+$, in a piece of dialysis membrane stretched around the bulb of the $NH_4^+$-sensitive glass electrode (see also chapter [41]). Prior to formation of the lactate dehydrogenase/glutamate dehydrogenase electrode the two enzymes, glutamate dehydrogenase (360 units), and lactate dehydrogenase (410 units), were combined and dialyzed against 50 m*M* Tris-HCl buffer (pH 8.0) containing 10 $\mu M$ Tris-EDTA and 100 $\mu M$ Tris-ADP to remove $NH_4^+$. The enzyme solution contained within the dialysis bag was then concentrated against sucrose to give a volume of 150–200 $\mu$l (compared to 350 $\mu$l before dialysis). Dextran-bound $NAD^+$ (25 mg dry weight) was dissolved in with the enzymes, and the resulting viscous solution was placed on a 5-cm square of dialysis membrane in contact with the tip of the electrode. After formation of the lactate dehydrogenase/glutamate dehydrogenase electrode about three-quarters of the enzyme solution remained entrapped by the membrane.

Immediately after its preparation and each time before use, the lactate dehydrogenase/glutamate dehydrogenase electrode was equilibrated in 50 m*M* Tris-HCl buffer (pH 8.0) made 10 $\mu M$ in Tris-EDTA and 100 $\mu M$ in Tris-ADP. When not in use, the enzyme electrode was kept at 4° in this same equilibration solution.

Glutamate and pyruvate concentrations were routinely determined in 50 ml of equilibration solution at 25°. Glutamate was determined in the presence of 2 m*M* pyruvate; pyruvate was measured in the presence of 10 m*M* glutamate. The millivoltage deflections caused by the serial addition of aliquots of 1 *M* glutamate or 0.2 *M* pyruvate to the stirred solutions were measured on a Radiometer ion meter (PHM 53) connected to a recorder. A standard fiber-junction saturated calomel electrode was used as a reference electrode.

As a control, the enzymes of the lactate dehydrogenase/glutamate

dehydrogenase electrode were inactivated by soaking the electrode in 8 *M* urea at 25° for 40 min. The electrode was then rinsed in distilled water and equilibrated in 50 m*M* Tris-HCl buffer (pH 8.0) to remove urea before being retested.

*Discussion*

As seen in Fig. 3a the millivoltage deflection of the electrode was directly proportional to the logarithm of the glutamate concentration in the range $10^{-4}$ to $10^{-8}$ *M*. Similar results were obtained in the determination of pyruvate (Fig. 3b). This enzyme electrode has potential application in the assay of 1-oxoglutarate and L-lactate by registering the uptake of $NH_4^+$ by the reverse of the above reactions (Scheme 1). Furthermore, other recycling systems comprising dextran-bound $NAD^+$ or $NADP^+$ could be envisaged for use as potential enzyme electrodes. Thus a system comprising coentrapped dextran-bound $NADP^+$, glucose-6-phosphate dehydrogenase, and glutamate dehydrogenase[5] might find application in the assay of physiological levels of glucose 6-phosphate.

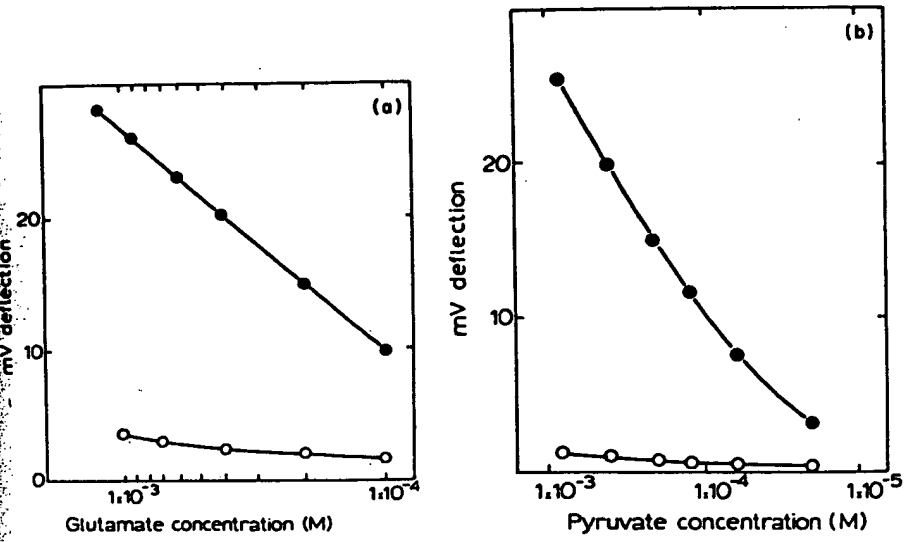



FIG. 3. Response curves for the enzyme/coenzyme electrode. (a) Estimation of glutamate using the lactate dehydrogenase/glutamate dehydrogenase electrode. (b) Estimation of pyruvate with the lactate dehydrogenase/glutamate dehydrogenase electrode.

A representative curve (●) made by plotting millivoltage deflection against (glutamate) and (pyruvate), respectively, is compared to a curve (○) obtained after denaturation of the lactate dehydrogenase/glutamate dehydrogenase electrode in 8 *M* urea. Reproduced with permission from P. Davies and K. Mosbach, *Biochim. Biophys. Acta* 370, 329 (1974).

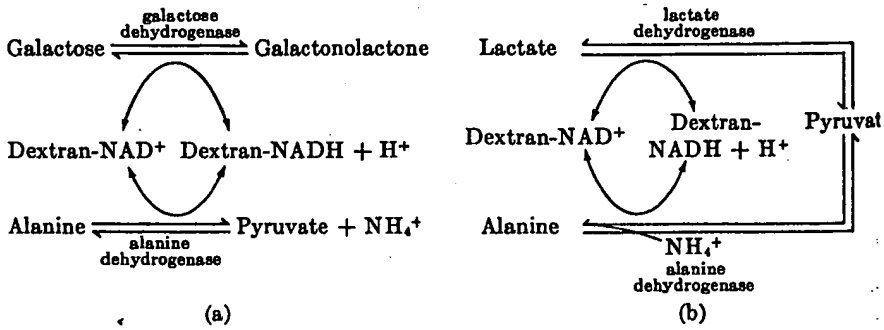
The response time of the lactate dehydrogenase/glutamate dehydrogenase electrode to additions of glutamate or pyruvate was approximately 3–4 min. The response of the electrode decreased by approximately 60% on storage at 4° over a period of 15 days although it was possible to recalibrate the electrode and determine metabolite concentrations as before. This instability of the enzyme electrode may reflect in part the inactivation of the enzymes themselves and in part their binding to the immobilized nucleotide. Clearly, refinements in the proportions of the components of the electrode and in stabilizing the enzymes could improve both the response time and the longevity of the system.

The use of immobilized coenzymes in enzyme electrodes considerably extends the range of potential substrates that can be assayed and, with careful optimization of the parameters discussed above, efficient and stable enzyme electrodes could be developed.

## "Enzyme Reactor"

The availability of enzymically regenerable immobilized coenzymes permits an extension in the scope of enzyme reactors (see in particular chapter [49] in this volume) from simple hydrolytic reactions to those such as dehydrogenases which utilize stoichiometric amounts of coenzymes in conversion of their substrates. Thus, addition of immobilized coenzymes obviates the economic burden of recovery of the coenzyme or of purifying the product.

Two model enzyme reactions[13] are described below to generate alanine from pyruvate by the action of alanine dehydrogenase. The two systems (Scheme 2a, b) differ in the enzymic means of generating dextran-bound NADH, with one utilizing galactose dehydrogenase (Scheme 2a) and the other lactate dehydrogenase (Scheme 2b).




SCHEME 2. Model reactor for production of alanine.

[13] P. Davies and K. Mosbach, *Biochim. Biophys. Acta* 370, 329 (1974).

## Example

### *Materials and Method*

The reaction mixture used in the enzyme reactor containing β-galactose dehydrogenase (2 units, Boehringer Corp., Tutzing, W. Germany) and L-alanine dehydrogenase (3 units) consisted of 50 m*M* sodium pyrophosphate buffer made 10 m*M* in galactose, 2 m*M* in sodium pyruvate, 200 m*M* in $NH_4Cl$, and 0.1 m*M* in EDTA. The mixture was adjusted to pH 8.8 with NaOH. The amount of dextran-bound $NAD^+$ analog was 10 mg dry weight.

The reaction mixture used in the other enzyme reactor containing beef-heart lactate dehydrogenase (85 units) and alanine dehydrogenase (24 units) consisted of 100 m*M* Tris base made 100 m*M* in L-lactic acid, 200 m*M* in $NH_4Cl$ and 0.01 m*M* in EDTA. The pH of this mixture was adjusted to 8.5. The amount of dextran-bound $NAD^+$ analog was 12 mg dry weight.

Pyruvate and alanine were determined spectrophotometrically using methods described by Lowry and Passonneau.[14] Rabbit muscle lactate dehydrogenase was used for the assay of pyruvate. Alanine was measured in a coupled assay by using glutamate-pyruvate transaminase in the presence of α-ketoglutarate to convert alanine to pyruvate, which in turn was assayed by using rabbit muscle lactate dehydrogenase. Alanine was determined in the presence of pyruvate by correcting for the contribution made to the reaction by the latter substance.

Enzyme reactor experiments were carried out in a Model 8 MC ultrafiltration apparatus (Amicon Corp., Lexington, Massachusetts) fitted with a PM 10 ultrafiltration membrane. The enzyme pairs together with dextran-bound $NAD^+$ were present in the small chamber of the apparatus in 2 ml of reaction mixture which contained the buffered substrates. Up to 100 ml more of the same reaction mixture were placed in the reservoir chamber. The ultrafiltration was done at 40–50 psi (which gives a flow rate of about 9 ml/hr) with the apparatus setting on "push liquid" such that the volume in the small chamber remained constant at 2 ml throughout the experiment. The filtrate was collected in 5-ml fractions for analysis.

### *Discussion*

The galactose dehydrogenase/alanine dehydrogenase reactor converted 30% of the added pyruvate (1.5 m*M*) to alanine at a constant

[14] O. H. Lowry and J. V. Passonneau, "A Flexible System of Enzymatic Analysis." Academic Press, New York, 1972.

rate over a period of 6.5 hr. In contrast, the conversion of pyruvate to alanine in the lactate dehydrogenase/alanine dehydrogenase reactor was initially 60–70% but fell to about 20% after 5.5 hr had lapsed. The immobilized $NAD^+$ was recycled at rates of 14 times per hour and 33 times per hour, respectively, within these reactors, assuming that all the $NAD^+$ was sterically available to the enzymes.

The two enzyme reactors described here represent model systems only. Clearly, with suitable adjustment of enzyme and dextran-bound $NAD^+$ concentrations and flow rate, a conversion rate to product approaching 100% could be realized.

### Applications in Enzymology and Protein Studies

To illustrate the wider potential of immobilized cofactors two examples of their application to fundamental studies will be given. In one case unmodified ATP was immobilized to Sepharose[15] using the cyanogen bromide method. The resulting preparation, of which the exact mode of binding of ATP remains to be established, was used to demonstrate the presence of an obligatory phosphoryl enzyme intermediate in the reaction catalyzed by succinyl-CoA synthetase. (The authors of this chapter recommend strongly the use of coenzyme analogs with functional groups suitable for binding like the ATP-analog described in this chapter.)

In another study, ATP bound to Sepharose was used to investigate the reaction between heavy meromyosin and ATP.[16] It was prepared by treating Sepharose with adipic or sebacic acid dihydrazide[16,17] and subsequently binding periodate-oxidized ATP. Both $Mg^{2+}$ and $Ca^{2+}$ activate the cleavage of bound ATP, some 40–50% of the total ATP being cleaved after 3.5 hr at 25°. Similarly, myosin and one-headed myosin[17] were bound to immobilized ATP, but only under conditions where, in the presence of $Ca^{2+}$ or $Mg^{2+}$, ATP was cleaved. Myosin could thus be resolved from modified myosin by virtue of differences in ATPase activity. In other words, binding is not a reversible process, but intimately associated with the occurrence of a chemical process. Similar studies using $N^6$-ADP-agarose derivatives have been reported.[18]

### Other Immobilized Coenzymes

Apart from the adenine nucleotide analogs described here, other derivatives have been reported where an intact demonstrably coenzy-

[15] E. A. Wider de Xifra, S. Mendiara, and A. M. del C. Batlle, *FEBS Lett.* 27, 275 (1972).
[16] R. Lamed, Y. Levin, and A. Oplatka, *Biochim. Biophys. Acta* 305, 163 (1973).
[17] R. Lamed and A. Oplatka, *Biochemistry* 13, 3137 (1974).
[18] I. P. Trayer, H. R. Trayer, D. A. P. Small, and R. C. Bottomley, *Biochem. J.* 139, 609 (1974).

mically active entity has been immobilized. One of the first reports describes the preparation of ε-aminohexanoyl-NAD-Sepharose obtained by carbodiimide coupling of $NAD^+$ to Sepharose substituted with ε-aminocaproic acid.[19] The coupling of $NAD^+$ to glass was published[20] independently. Both preparations, however, showed low coenzymic activity and since the latter preparation showed poor stability, another derivative, $N^6$-aminoethyl, was synthesized by a similar alkylation step to that described in this chapter using aziridine.[21] In its immobilized dextran form, this preparation showed 2–10% of the maximum turnover number ($k_{cat}$) of unmodified $NAD^+$. Whereas to our knowledge no applications of this $NAD^+$ derivative have been reported, another $NAD^+$ derivative, $N^6$-succinyl-$NAD^+$, coupled to a polyethyleneimine carrier has been applied in a two-enzyme recycling system[22] comprising alcohol dehydrogenase and lactate dehydrogenase. Furthermore, this immobilized $NAD^+$ derivative was shown to be reducible by alcohol dehydrogenase bound to aminoethyl cellulose. Preliminary studies with this analog bound to aminodextran and present in a hollow fiber have also been reported.[23] However, the somewhat dubious stability of this acyl-linked derivative of $NAD^+$ above pH 7 seems to limit its general applicability. In contrast, the more stable alkyl linkage of the dextran-bound $NAD^+$ derivative described in detail here seems to make it more suitable for use in prospective enzyme reactors.

Other recently synthesized $NAD^+$ analogs which have been successfully applied in affinity chromatography should also be coenzymically active. These are a nicotinamide-6-mercaptopurine dinucleotide[24] as well as $N^6$-(6-aminohexyl)-$NAD^+$[6]. All the above derivatives except for one[20] are either substituted at the $N^6$-position or at "*S*.[6]" Substitution at position 8 in the adenine moiety, as in the 8-(6-aminohexyl)-amino derivatives of cAMP,[9] AMP,[25,26] and in particular $NAD^+$[26] and $NADP^+$,[27] pro-

[19] P. O. Larsson and K. Mosbach, *Biotechnol. Bioeng.* **13**, 393 (1971).
[20] M. K. Weibel, H. H. Weetall, and H. J. Bright, *Biochem. Biophys. Res. Commun.* **44**, 347 (1971).
[21] M. K. Weibel, C. W. Fuller, J. M. Stadel, A. F. E. P. Buckmann, T. Doyle, and H. J. Bright, *in* "Enzyme Engineering" (E. K. Pye and L. B. Wingard, eds.), Vol. 2, p. 203. Plenum, New York, 1974.
[22] J. R. Wykes, P. Dunnill, and M. D. Lilly, *Biochim. Biophys. Acta* **286**, 260 (1972).
[23] R. P. Chambers, J. R. Ford, J. H. Allender, W. H. Baricos, and W. Cohen, *in* "Enzyme Engineering" (E. K. Pye and L. B. Wingard, eds.), Vol. 2, p. 195. Plenum, New York, 1974.
[24] S. Barry and P. O'Carra, *FEBS Lett.* **37**, 134 (1973).
[25] B. Jergil, H. Guilford, and K. Mosbach, *Biochem. J.* **139**, 441 (1974).
[26] C.-Y. Lee, D. A. Lappi, B. Wermuth, J. Everse, and N. O. Kaplan, *Arch. Biochem. Biophys.* **163**, 561 (1974).
[27] C.-Y. Lee and N. O. Kaplan, *Arch. Biochem. Biophys.* **168**, 665 (1975).

vide alternative choices as do other related 8-substituted $NAD^+$ derivatives.[28]

The immobilization of several coenzymes and coenzyme analogs for affinity chromatography has been described in detail in this series[1] and elsewhere,[29,30] and in the following only those examples where an intact, demonstrably coenzymically active entity other than $NAD^+$, $NADP^+$, or ATP has been immobilized will be considered here.

Adenosine-3′,5′-cyclic monophosphate (cAMP) has been attached to Sepharose and shown to be effective for the biospecific adsorption of the cAMP-stimulated protein kinases.[25,31] The cAMP seems to act by promoting dissociation of the enzyme into a regulatory, catalytically inactive, cAMP-binding subunit and an enzymically active catalytic subunit.[32,33] Resolution of the catalytic and regulatory subunits can be accomplished on casein- or histone-Sepharose columns[34,35] or on cAMP columns.[25,31] Thus, protein kinase from trout testis (protamine kinase)[34,35] could be eluted from 8-(6-aminohexyl)amino-cAMP-Sepharose by a pulse of the inhibitor AMP, but was no longer activated by cAMP.[25]

Flavin mononucleotide (FMN) has been coupled to hexanoyl-Sepharose with dicyclohexylcarbodiimide in aqueous pyridine[36] by a procedure essentially analogous to that described for the preparation of hexanoyl-$NAD^+$-Sepharose.[19] The resulting immobilized FMN binds bacterial luciferase and when reduced, either catalytically with $H_2$ or with dithionite, is effective as a substrate in the luminescence reaction. The maximum rate of oxidation of immobilized $FMNH_2$ was only approximately 2% of that of free $FMNH_2$, although, surprisingly, the turnover time for the reaction initiated by the immobilized $FMNH_2$ was identical to the soluble flavin, using both decanal and dodecanal as aldehyde cosubstrates. Substitutions on the flavin molecule and the chain length of the aldehyde generally significantly influence the turnover time. The low rate of enzymic oxidation of $FMNH_2$-Sepharose by bacterial

[28] P. Zappeli, A. Rossodivita, G. Prosperi, R. Pappa, and L. Re, *Eur. J. Biochem.* **62**, 211 (1976).
[29] C. R. Lowe and P. D. G. Dean, "Affinity Chromatography," pp. 92–129. Wiley, New York, 1974.
[30] K. Mosbach, *Biochem. Soc. Trans.* **2**, 1294 (1974).
[31] M. Wilchek, Y. Salomon, M. Lowe, and Z. Sellinger, *Biochem. Biophys. Res. Commun.* **45**, 1177 (1971).
[32] G. N. Gill and L. D. Garren, *Biochem. Biophys. Res. Commun.* **39**, 335 (1970).
[33] M. Tao, M. L. Salas, and F. Lipmann, *Proc. Natl. Acad. Sci. U.S.A.* **67**, 408 (1970).
[34] J. D. Corbin, C. O. Brostrom, R. L. Alexander, and E. G. Krebs, *J. Biol. Chem.* **247**, 3736 (1972).
[35] J. D. Corbin, C. O. Brostrom, C. A. King, and E. G. Krebs, *J. Biol. Chem.* **247**, 7790 (1972).
[36] C. A. Waters, J. R. Murphy, and J. W. Hastings, *Biochem. Biophys. Res. Commun.* **57**, 1152 (1974).

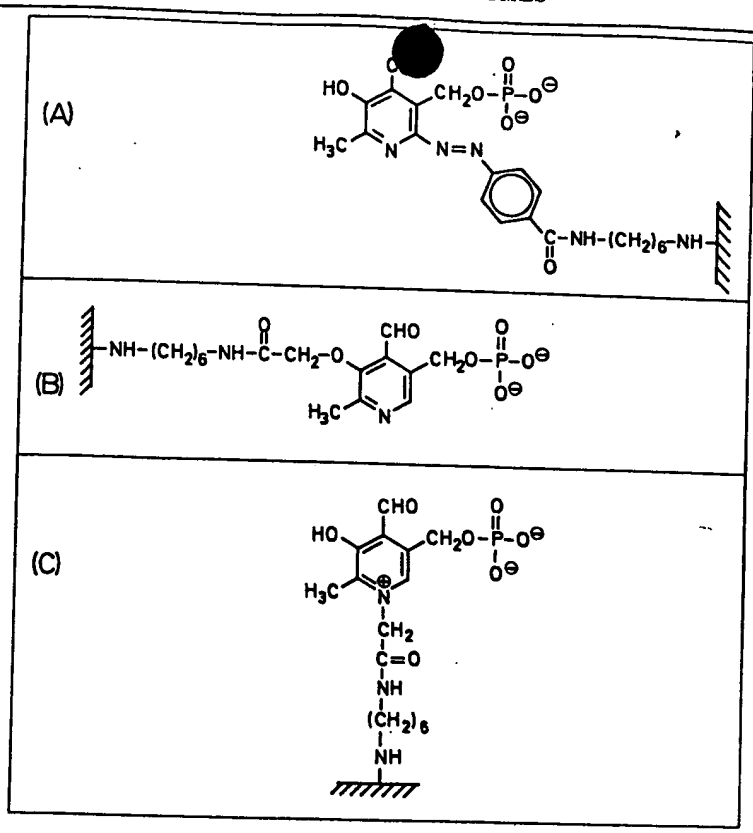



FIG. 4. Three immobilized pyridoxal 5′-phosphate derivatives. (A) 6-Immobilized diazo derivative; (B) 3-O-immobilized; and (C) N-immobilized.

luciferase may possibly be ascribed to the steric restraints imposed by immobilization and to the derivatization of FMN with a hexanoyl group.

Pyridoxal 5′-phosphate has been coupled to diazotized *p*-aminobenzamidohexyl-Sepharose[37] and catalyzes the cleavage of tryptophan in the presence of $Cu^{2+}$, as does free pyridoxal 5′-phosphate. This system can be utilized for the immobilization of the enzyme since the immobilized pyridoxal 5′-phosphate retained functional groups for binding to the apoproteins of $B_6$-dependent enzymes. The resulting pyridoxal 5′-phosphate-Sepharose-apotryptophanase complex was reduced with sodium borohydride to generate a derivative which retained approximately 60% of the catalytic activity of the free tryptophanase used.

This approach was later extended[38] with the introduction of two other Sepharose-bound analogs (Fig. 4). Both N-immobilized and 3-O-

[37] S.-I. Ikeda and S. Fukui, *Biophys. Res. Commun.* 52, 482 (1973).
[38] S.-I. Ikeda, H. Hara, and S. Fukui, *Biochim. Biophys. Acta* 372, 400 (1974).

immobilized pyridoxal 5′-phosphate were synthesized by reaction of pyridoxal 5′-phosphate with a bromoacetyl derivative of Sepharose in 50% (v/v) dimethylformamide and in potassium phosphate buffer, pH 6.0, respectively, for approximately 70 hr at room temperature. The catalytic activities of these derivatives were tested in the nonenzymic cleavage of tryptophan. The N-immobilized pyridoxal 5′-phosphate analog displayed catalytic activity, but the 3-O-immobilized derivative did not exhibit appreciable activity. This behavior, however, is not reflected in the ability of the two derivatives to bind apotryptophanase. Alkylation of the pyridine nitrogen of pyridoxal 5′-phosphate leads to a decreased affinity for the apoprotein, but attachment to Sepharose via the 3-hydroxyl group does not impede binding since this analog maintains all the main functional groups necessary for binding to the apoenzyme. Both derivatives bind apotryptophanase in such a way as to retain 50–60% of the specific activity of the starting material. Tyrosine phenol-lyase ($\beta$-tyrosinase) from *Escherichia intermedia* has been similarly immobilized.[39] This method of enzyme immobilization was found to be superior to other methods commonly used for preparation of immobilized enzymes.

## General Discussion

In summarizing, the following can be said. Two conditions for the practical application of enzyme systems requiring expensive cofactors have to be met, i.e., their retention and regeneration. The regeneration may be accomplished either enzymically as described here, chemically with the participation of artificial electron acceptors or donors,[28] or electrochemically[40] (see also chapter [58] this volume). Depending on the type of application, either one of these may be the best choice. Enzymic regeneration appears to be the procedure of choice in the application of enzyme–coenzyme systems in the potential treatment of enzyme deficiency diseases whereby the catalyst system may be used entrapped in microcapsules or polymer beads and which are either placed in extracorporal shunts or implanted *in vivo*.

With regard to retention, it appears advantageous to have the cofactors immobilized to macromolecular supports although attempts have been made to use unmodified $NAD^+$ in hollow fibers as well.[28] However,

[39] S. Fukui, S.-I. Ikeda, M. Fujimura, H. Yamada, and H. Kumagai, *Eur. J. Biochem.* 51, 155 (1975).

[40] M. Aizawa, R. W. Coughlin, and M. Charles, *Biochim. Biophys. Acta* 385, 362 (1975).

the requirement for tight membranes to keep the $NAD^+$ entrapped leads to overall poor permeability for substrate/product. Another possibility should be mentioned: for some applications, such as medical and some analytical procedures, which do not require a separation of the catalyst system, including the coenzyme, from the solution in which they are applied, enzymic recycling of the unmodified coenzyme may be the procedure of choice provided the number of cycles is high enough.

Finally an additional possible solution to the problem of coenzyme retention may be found with the following approach. In a previous study glycogen phosphorylase *b* has been immobilized in its allosterically activated form.[41] This had been accomplished by coimmobilization of the enzyme and its positive allosteric effector, AMP, using the AMP analog $N^6$-(6-aminohexyl)-AMP, to CNBr-activated Sepharose. In extending these studies an alcohol dehydrogenase–NAD(H)–Sepharose complex was prepared showing no requirement of soluble coenzyme for its activity, at the same time with the coenzyme susceptible to recycling.[42] This was accomplished in a similar fashion by coupling a preformed enzyme–coenzyme binary complex to an activated matrix.

[41] K. Mosbach and S. Gestrelius, *FEBS Lett.* **42**, 200 (1974).
[42] S. Gestrelius, M. O. Månsson, and K. Mosbach, *Eur. J. Biochem.* **57**, 529 (1975).

## [62] Covalent Immobilization of Adenylate Kinase and Acetate Kinase in a Polyacrylamide Gel: Enzymes for ATP Regeneration[1]

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Adenylate kinase (AMP:ATP phosphotransferase, EC 2.7.4.3) and acetate kinase (ATP:acetate phosphotransferase, EC 2.7.2.1) form the basis for a procedure for the regeneration of ATP from AMP and/or ADP, using the readily available acetyl phosphate (AcP)[2] as the ultimate phosphorylating agent.[3] Both adenylate kinase and acetate kinase

[1] Supported by the National Science Foundation, (RANN) Grant GI 34284.
[2] G. M. Whitesides, M. Siegel, and P. Garrett, *J. Org. Chem.* **40**, 2516 (1975).
[3] C. R. Gardner, C. K. Colton, R. S. Langer, B. K. Hamilton, M. C. Archer, and G. M. Whitesides, *in* "Enzyme Engineering" (E. K. Pye and L. B. Wingard, Jr., eds.), Vol. 2, p. 209. Plenum, New York, 1974; G. M. Whitesides, A. Chmurny, P. Garrett, A. Lamotte, and C. K. Colton, *ibid.*, p. 217.